Phenolics and Betacyanins in Red Beetroot (*Beta vulgaris*) Root: Distribution and Effect of Cold Storage on the Content of Total Phenolics and Three Individual Compounds

Tytti S. Kujala,* Jyrki M. Loponen, Karel D. Klika, and Kalevi Pihlaja

Department of Chemistry, University of Turku, FIN-20014 Turku, Finland

The distribution of total phenolics and main betacyanins in red beetroot (*Beta vulgaris*) root was determined. Also, the subsequent effects of cold storage on the content of total phenolics, main betacyanins (betanin and isobetanin), and the main known ferulic acid ester (*β*-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) were determined in the peel, which is the root part containing the largest amount of total phenolics. The content of total phenolics in the red beetroot water extracts was determined according to a modification of the Folin−Ciocalteu method and expressed as gallic acid equivalents (GAE). The compounds of interest were identified by HPLC−ESI−MS and NMR techniques, and the contents of compounds were determined by HLPC analyses. The total phenolic contents in various root parts were found to decrease in the order peel, crown, flesh. Significant differences in the contents of total phenolics and individual compounds were found when the effect of cold storage (5 °C, 0−196 days) on the constituents of the peel from intact roots was examined. In addition to the betacyanins of red beetroot peel found in our earlier study, tentative identifications of betanidin and feruloylamaranthin were made.

Keywords: *Beta vulgaris; red beetroot; phenolics; betacyanins; distribution of phenolics; distribution of betacyanins; effect of cold storage*

INTRODUCTION

The suggested advantageous health effects (maintenance of health and protection from diseases such as cancer and coronary heart disease) of plant phenolics and the possibility to use antioxidant plant constituents as food ingredients has motivated plant phenolics research. Interest in the phenolic composition and antioxidant action of fruit and vegetable extracts has increased recently (Al-Saikhan et al., 1995; Cao et al., 1996; Wang et al., 1996; Velioglu et al., 1998; Vinson et al., 1998; Kähkönen et al., 1999). Because of the nutritional importance of plant phenolics, in the past few years there has been an increasing interest in the evaluation of their changes with postharvest treatments (Crozier et al., 1997; Friedman, 1997; Chaudry et al., 1998; Gil et al., 1998; Lewis et al., 1999).

Red beetroot (*Beta vulgaris*) concentrate is universally permitted as a food ingredient as beetroot red; thus, investigations of red beetroot constituents concentrate on the betalains (red-violet betacyanins and yellow betaxanthins). The reported exceptional antioxidant activities of beet extracts has increased the interest in red beetroot compounds. Vinson et al. (1998) ranked beets among the ten most potent vegetables with respect to antioxidant activity. Peel extract of both red beetroot and sugar beet showed strong antioxidant activity in our earlier study, when 92 plant extracts of Finnish origin were screened with respect to their total phenolic content and antioxidant activity (Kähkönen et al., 1999). Zakharova and Petrova (1998) have investigated the antioxidant activity of certain betalains, and Escribano et al. (1998) have characterized the antiradical activity of betalains from red beetroot.

Betalains and anthocyanins are mutually exclusive in their natural occurrence, but other flavonoids (e.g., flavonols and flavones) are often produced in betalain-bearing plants (Stafford, 1994). Phenolic acids and phenolic acid conjugates have also been reported in different beet materials (Bokern et al., 1991; Waldron et al., 1997; Ng et al., 1998; Harborne et al., 1999; Wende et al., 1999).

We started the investigation of red beetroot *(Beta vulgaris*) peel constituents by characterizing chemical compounds of fractionated red beetroot peel extract. Several betacyanins (betanin, prebetanin, isobetanin, and neobetanin), *p*-coumaric acid, ferulic acid, and traces of unidentified flavonoids were found (Kujala et al., unpublished results). We also studied the effect of extraction method on the composition of extract. In addition to the extraction method, other factors influence the constituents of plant extract: postharvest treatments and factors generating intraspecific differences in plants (age, phenological stage, disease, etc.) (Waterman and Mole, 1994). The purposes of the present study were to determine the distribution of total phenolics and main betacyanins in red beetroot (*Beta vulgaris*) root, and to determine the effect of cold storage on the content of total phenolics, two main betacyanins (betanin and isobetanin), and the main known ferulic acid ester (*β*-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) of the peel (which is the root part containing the largest amount of total phenolics). Structures of the individual compounds studied (betanin (**I**), isobetanin (**II**), and *β*-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) (**III**)) are shown in Figure 1.

* To whom correspondence should be addressd. Department of Chemistry, University of Turku, Vatselankatu 2, FIN-20014, Turku, Finland. Telephone: +358 2 3336828. Fax: +358 2 3336700. E-mail: tytti.kujala@utu.fi.

10.1021/jf000523q CCC: $19.00
Published on Web 10/07/2000

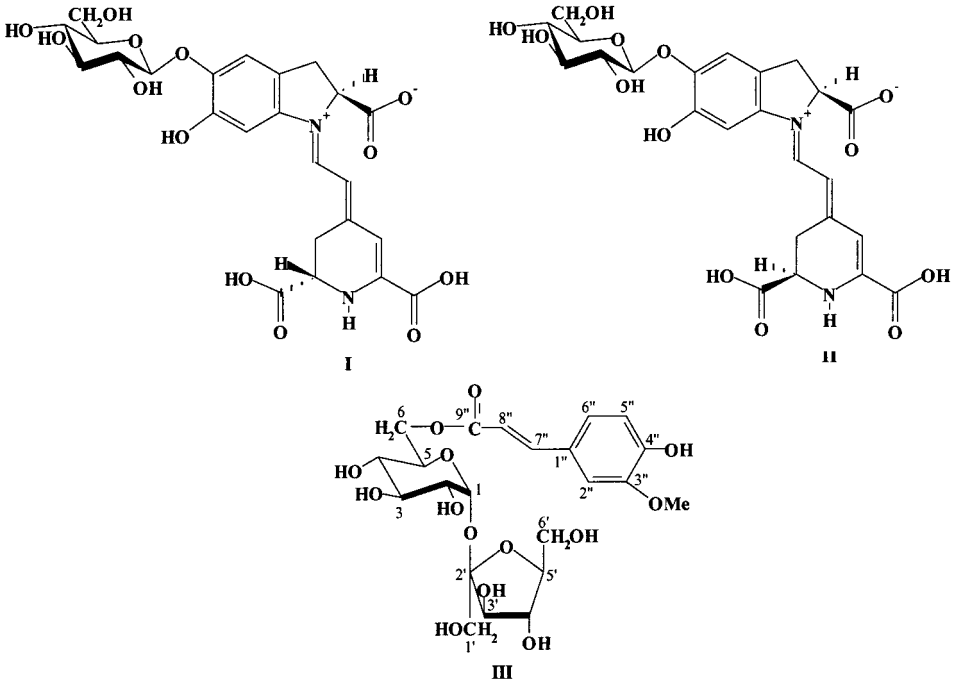

Figure 1. Structures of betanin (**I**), isobetanin (**II**), and β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) (**III**).

MATERIALS AND METHODS

Chemicals. Folin−Ciocalteu's phenol reagent was purchased from Fluka (Biochemica, Buchs, Switzerland), sodium carbonate was purchased from Merck (Darmstadt, Germany), and ferulic acid was purchased from Sigma (St. Louis, MO). All organic solvents used were of HPLC grade.

Plant Material. The red beetroots (*Beta vulgaris,* var. Little Ball) studied were harvested in September 1998 in Salo, Finland. The beets were stored in the dark in a cold cellar (5 °C) until sampling (after 0, 35, 63, 98, 140, 168, and 196 days of storage, ca. 2 kg of red beetroots/sampling). The sampled beets were washed and hand-peeled, and the collected peels were cut into small pieces and stored at −25 °C until lyophilization. From the first sample of red beetroots, the flesh and the crown were also separated and treated similarly to the peel. Lyophilized plant material was reduced into powder with a mortar and pestle. A portion of the lyophilized beetroot peel was stored at −20 °C for 9 months for comparative analysis.

Extraction. From each studied plant material five replicate extracts were prepared. Lyophilized plant material (500 mg) was homogenized (Ultra-Turrax) for one minute with 10 mL of water. The homogenate was centrifuged for 10 min (1500*g*), and the clear supernatant was collected. The insoluble part was re-extracted with 10 mL of water. The extracts were combined and the final volume of the extract was made up to 15.5 mL.

Determination of Total Phenolics. The amount of total phenolics in the extracts was determined according to a modification of the Folin−Ciocalteu method (Nurmi et al., 1996). A 1.0-mL aliquot of diluted extract (extract−water, 1:40 (v/v), two replicates) was introduced into a test tube and mixed with 1.0 mL of 1 N Folin−Ciocalteu's reagent. The mixture was allowed to stand for a 2 to 5 min period which was followed by the addition of 2.0 mL of 20% Na_2CO_3. After 10 min incubation at room temperature, the mixture was centrifuged for 8 min (150*g*) and the absorbance of the supernatant was measured at 730 nm on a Perkin-Elmer Lambda 12 UV/VIS Spectrophotometer (Norwalk, CT). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material.

HPLC Analysis. Analytical chromatographic measurements were performed on an HPLC system consisting of a Merck-Hitachi L-7200 autosampler, Merck-Hitachi L-7100 HPLC pump, Merck-Hitachi L-7400 UV/VIS detector, and Merck-Hitachi D-7000 interface (all from Hitachi, Tokyo, Japan). The column used was a 5-μm Purospher RP-18 (250 × 4.6 mm i.d., Merck, Darmstadt, Germany) equipped with a precolumn. The injection volume was 20 μL and the constant flow rate was 1.0 mL min^{-1}. Two solvents, acetonitrile (A) and formic acid−water (5:95, v/v) (B) were used. The elution profile was 0−5 min, 100% B (isocratic); 5−50 min, 0−20% A in B (linear); 50−60 min, 20−70% A in B (linear). The detector wavelength was 280 nm.

Quantification. I and **II** were quantified by comparison with an external standard of betanin (isolated from the extracts) and **III** as ferulic acid.

Isolation of I and III. Compounds **I** and **III** were isolated from the extracts semipreparatively (LiChroprep RP-18, 40−63 μm; Merck, Darmstadt, Germany) eluting different solutions of acetonitrile and formic acid−water (0.4:99.6, v/v). Isolated **I** was used to quantification and **III** was subjected to MS and NMR analyses.

Identification of the Compounds. The structures of the studied compounds were confirmed by comparison with literature data (Strack et al., 1988; Bokern et al., 1991; Jackman and Smith, 1996).

HPLC−ESI−MS and ESI−MS/MS Analysis. High-performance liquid chromatographic−electrospray ionization−mass spectral (HPLC−ESI−MS) and ESI−MS/MS analyses of the extracted and isolated compounds of red beetroot peel were conducted using a Perkin-Elmer Series 200 HPLC system and SCIEX API 365 triple-quadrupole mass spectrometer (PE Sciex, Toronto, Canada). Spectral and chromatographic data were collected on an Apple Macintosh 8.1 Data system. The HPLC system consisted of two Perkin-Elmer Series 200 micro pumps (Perkin-Elmer, Norwalk, CT) and a 785A UV/VIS detector (Perkin-Elmer, Norwalk, CT). Samples were introduced into the system by loop injection from a Perkin-Elmer Series 200 Autosampler (Perkin-Elmer, Norwalk, CT). The eluents were acetonitrile (A) and formic acid−water (0.4:99.6, v/v) (B). The gradient profile was the same as above. The flow was split prior to the ion source. For ESI−MS/MS, sample (in 50% methanol−water solution) was introduced by a direct infusion pump (Harvard Apparatus, Saint-Laurent, Quebec, Canada) at a constant flow rate 0.4 mL hr^{-1}.

Electrospray ionization was used for MS analysis. For HPLC−ESI−MS analysis the mass spectrometer was operated in both the negative and positive modes, and the masses were scanned from *m*/*z* 100 to 1100 in 0.3 amu steps. For negative

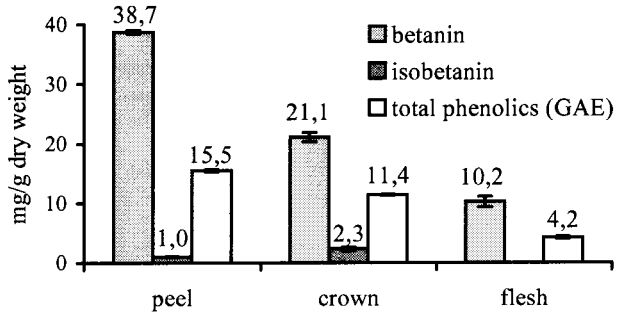

Figure 2. Betanin (**I**), isobetanin (**II**), and total phenolic (GAE) contents (mean ± SE) in red beetroot peel, crown, and flesh.

ion measurements the spray needle voltage was set at −4000 V; the orifice voltage was set at −35 V and the ring voltage was set at −220 V. The setting for purified air nebulizer-gas flow was 9 and for the curtain-gas (N_2) flow was 12. For positive ion experiments the needle voltage was 5200 V; orifice voltage was set at 45 V; ring voltage was 220 V. Nebulizer and curtain gas flow settings were 8 and 10, respectively. The heated nitrogen gas temperature was set at 300 °C. Conditions for ESI−MS/MS analyses with negative polarity were needle voltage, −4000 V; orifice, −41; ring, −210; and collision gas energy, 25 V. Nebulizer, curtain, and collision gas flow settings were 8, 12, and 3, respectively. Scan range was from *m/z* 100 to 520 with 0.2-amu step sizes.

NMR Analysis. NMR spectra were acquired on a JEOL Lambda 400 NMR spectrometer equipped with a 5-mm normal configuration tunable probe operating at 399.78 MHz for ^{1}H and 100.44 MHz for ^{13}C. The spectra were recorded at 25 °C in CD_3OD. ^{1}H spectra were referenced internally to tetramethylsilane (0 ppm) and ^{13}C spectra were referenced internally to the solvent (49.15 ppm). 1D proton spectra were acquired with single-pulse excitation, 80° flip angle, pulse recycle time of 8 s, and with spectral widths of 8 kHz consisting of 64 k data points (digital resolution 0.12 Hz/pt), zero-filled to 128 k prior to Fourier transformation. 1D carbon spectra were acquired with single-pulse excitation, 45° flip angle, pulse recycle time of 3.5 s, and with spectral widths of 34 kHz consisting of 64 k data points (digital resolution 0.52 Hz/pt), zero-filled to 128 k and with 1 Hz exponential weighting applied prior to Fourier transformation. DEPT 135° spectra were acquired with similar spectral windows and with a pulse delay time of 3 s. Assignments of both spectra were based on literature values, and the multiplicities of the carbon spectra were confirmed by DEPT 135° measurements.

Statistical Analysis. All values given are the average of five replicates. Significance of the effect of cold storage was analyzed by t-test. The correlations between the total content of phenolics and the contents of individual phenolics were tested with the Pearson correlation coefficient.

RESULTS

Distribution of Phenolics and Betacyanins. The phenolic compounds are distributed mostly to outer parts of the root of red beetroot (*Beta vulgaris*). The total phenolic content and the betanin (**I**) content in various root parts were found to decrease in the order peel, crown, flesh. Detectable levels of isobetanin (**II**) were found in the crown and the peel, but not in the flesh. Data for the amounts of total phenolics, **I**, and **II** are presented in Figure 2. A positive correlation (Pearson correlation coefficient = 0.98) was found between the amounts of betanin (**I**) and total phenolics.

Effect of Cold Storage. Significant differences ($P < 0.0001$) in the amounts of betanin (**I**), isobetanin (**II**), β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) (**III**), and total phenolics were noticed when the effect of cold storage (5 °C, 0−196 days) was studied. The amount of **I** in peels of cold-stored red beetroots ranged from 38.7 ± 0.3 to 15.5 ± 0.4 mg/g; the amount of **I** decreased until 140 days of storage, after which the level began to rise. The amount of **II** ranged from 1.0 ± 0.04 to 4.8 ± 0.3 mg/g. The β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) (**III**) content was noticed to increase with the storage time, and the amount ranged from the detection limit to 357.7 ± 6.7 μg/g. The amount of total phenolics ranged from 15.5 ± 0.1 to 13.1± 0.3 mg GAE/g; it decreased steadily until 63 days of storage and the changes after that were minor. A positive correlation (Pearson correlation coefficient = 0.92) was found between the amounts of betanin (**I**) and total phenolics. Data for the amounts of total phenolics and the contents of **I**, **II**, and **III** are presented in Figure 3.

The amounts of **I** and **II** decreased significantly ($P < 0.0001$) in lyophilized red beetroot peel during the cold storage (−20 °C, 9 months) (Figure 4). No significant change was noticed in the total phenolic content. The amounts of betacyanins (**I** and **II**) were found to correlate positively (Pearson correlation coefficient = 1) with the amount of total phenolics.

The Compounds. In addition to our earlier study on the constituents of red beetroot peel (Kujala et al., unpublished results) the identification of **III** and tentative identification of betanidin and feruloylamaranthin were made.

HPLC−ESI−MS Analysis. **I** and **II** (MW:s 550) showed $[M + H]^+$ and $[M - H]^-$ ions in the positive and negative modes at *m/z* 551 and 549, respectively. In addition, both showed a significant fragment ion at *m/z* 389 in positive mode. This was attributed to $[\text{betanidin} + H]^+$. A compound, eluting after **I** and **II**, with $[M + H]^+$ ion at *m/z* 389, was tentatively identified as betanidin (MW, 388). The tentative identification of feruloylamaranthin (MW, 903) was based on the $[M + H]^+$ ion at *m/z* 904 together with a fragment ion at *m/z* 550 [M − feruloylglucuronosylglucoside + H]$^+$.

III yielded only $[M - H]^-$ ion at *m/z* 517 in the negative mode. MS/MS of the $[M - H]^-$ ion at *m/z* 517 from isolated material yielded the fragment ion at *m/z* 355, indicating the loss of 162 amu ion. This was attributed to the loss of the fructose residue $[M - H - \text{Fruc}]^-$. The ion at *m/z* 193, characteristic for a feruloyl residue, indicates the loss of both fructose and glucose residues (162 amu for both) from $[M - H]^-$ ion.

*The NMR Spectral Data for **III**.* ^{13}C NMR δ: 147.22 (s × 2, C3″, C4″), 124.44 (d, C6″), 116.59, 115.49 (d × 2, C5″, C7″), 111.81 (d, C2″), 105.36 (s, C2′), 93.41 (d, C1), 84.05 (d, C5′), 79.38 (d, C3′), 76.22 (d, C4′), 74.77 (d, C3), 73.35 (d, C2), 72.20 (d, C5), 72.03 (d, C4), 65.26 (t, C6), 64.38 (t × 2, C1′, C6′), 56.63 (q, C10″). Two quaternary carbons, C9″ and C1″, were not detected because of poor sensitivity arising from insufficient sample. ^{1}H NMR δ: 7.635 (d, $J_{8''} = 15.9$ Hz, H7″), 7.233 (d, $J_{6''} = 1.9$ Hz, H2″), 7.092 (dd, $J_{5''} = 8.4$, $J_{2''} = 1.9$ Hz, H6″), 6.804 (d, $J_{6''} = 8.2$ Hz, H5″), 6.436 (d, $J_{7''} = 15.9$ Hz, H8″), 5.413 (d, $J_2 = 3.8$ Hz, H1), 4.497 (dd, $J_{6B} = 11.9$, $J_5 = 2.0$ Hz, H6A), 4.261 (dd, $J_{6A} = 11.9$, $J_5 = 6.2$ Hz, H6B), 4.111 (ddd, $J_4 = 10.1$, $J_{6B} = 6.1$, $J_{6A} = 1.9$ Hz, H5), 4.094 (d(AB), $J_{4'} = 8.4$ Hz, H3′), 4.051 (d(AB)d, $J_{3'} = 8.4$, $J_{5'} = 7.5$ Hz, H4′), 3.890 (3H s, H10″), 3.88−3.71 (4H overlapped mult., H6′A, H5′, H6′B, H3), 3.621 (d(AB), $J_{1'B} = 12.3$ Hz, H1′A), 3.596 (d(AB), $J_{1'A} = 12.3$ Hz, H1′B), 3.452 (dd, $J_3 = 9.8$, $J_1 = 3.8$ Hz, H2), signal of H4 overlapped with solvent.

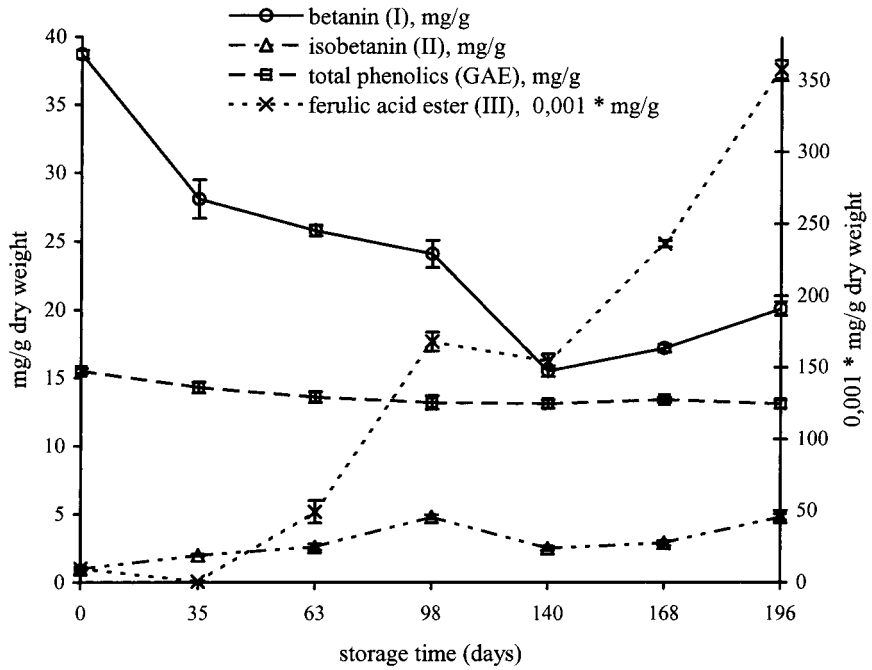

Figure 3. Effect of cold storage (5 °C) on betanin (**I**), isobetanin (**II**), total phenolic (GAE), and β-D-fructofuranosyl-α-D-(6-O-(*E*)-feruloylglucopyranoside) (**III**) contents (mean ± SE) of red beetroot peel.

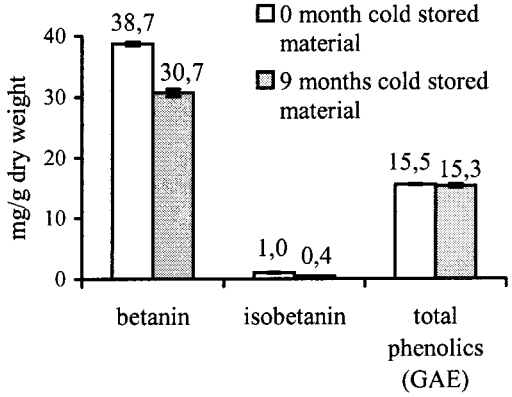

Figure 4. Betanin (**I**), isobetanin (**II**), and total phenolic (GAE) contents (mean ± SE) for 0- and 9-months-stored lyophilized red beetroot peel.

DISCUSSION

Distribution of Phenolics and Betacyanins. The composition and distribution of phenolic compounds varies widely in the plant kingdom and within the plant. The total phenolics distribution in red beetroot (*Beta vulgaris*) root appears to be quite similar to that reported for the potato. In potato, the phenolic compounds are distributed mostly between the cortex and skin (peel) tissues accounting for about 50% of the phenolics, and the remainder decreases in concentration on going from the outside toward the center of the potato tubers (Friedman, 1997).

Betalains (betacyanins and betaxanthins) have been detected only in red-violet-, orange-, and yellow-pigmented botanical species belonging to closely related families of the order Caryophyllales. Betalains accumulate in cell vacuoles of the flowers, fruits, and leaves of the plants that synthesize them, mainly in epidermal and/or subepidermal tissues (Jackman and Smith, 1996). This localized accumulation of betalains was shown also in our study.

Effect of Cold Storage. Even though the positive correlation (Pearson correlation coefficient = 0.92) between the amount of betanin (**I**), the main phenolic compound in red beetroot, and the amount of total phenolics was found, the effect of cold storage on the total phenolic content of red beetroot peel was minor in comparison to differences found between individual compounds. It is apparent that the total phenolics method does not give a full picture of the quantity or quality of the phenolic constituents in water extracts of red beetroot. Several authors have emphasized the importance of the extraction method in determining the types of compounds that will be obtained, because different compounds have different responses for the methods, and the extract may contain nonphenolic compounds, which absorb in the ultraviolet (Torres et al., 1987). It is possible that the decreases and increases of phenolics cancel one another and thus cover the real change in the total phenolic content: e.g., the amount of **I** decreased and the amounts of **II** and **III** increased until 98 days of storage (Figure 3). The large changes in betanin content could be explained by the poor stability of the compound. It should also be noticed that not all the degradation products of betanin are phenolic. The increase of betanin content after 140 days of storage could be rationalized by the ability of red beet pigments to degrade and regenerate continuously during storage, as the reaction is reversible (Han et al., 1998).

The effects of different kinds of storage methods on the phenolic composition in plants have been studied and, like in our study, both decreases and increases in phenolic contents have been reported. For example, Chaudry et al. (1998) studied the phenolic compounds of solar-cabinet-dried persimmon during storage and noticed a decreasing trend of phenolics; Lewis et al. (1999) reported an increase in the anthocyanin, flavonoid, and total phenolic acid concentrations of colored potato tubers during cold storage (4 °C).

Several factors affect the stability of betalains during processing and storage: storage temperature, pH, type of buffer solution, and the presence or absence of oxygen

(Han et al., 1998). Osornio and Chaves (1998) studied the quality changes in stored raw grated beetroots as affected by temperature and packaging film, and reported a notable decrease in the pigment content during storage. Pátkai et al. (1997) reported the following retention of betanine during nectar production: (1) raw material, 100%; (2) blanching, 99.8%; (3) peeling, 99.4%; (4) crushing, homogenization, filling, 91.6%; (5) pasteurization, 50.1%; (6) storage (20 °C, 60 days) 31.3%; and (7) storage (5 °C, 60 days), 46.9%. Our results on the effect of up to 140 days of cold storage on the red beetroot peel betanin content resemble these reported decreases in the pigment contents of beetroot materials.

The Compounds. Most varieties of red beetroot contain betanin (5-*O*-*β*-D-glucopyranosylbetanidin) as the predominant coloring, and this represents 75 to 90% of the total color present (Henry, 1996). Other betacyanins found in different parts and cell cultures of red beetroot are betanidin, isobetanidin, isobetanin (5-*O*-*β*-D-glucopyranosylisobetanidin), prebetanin (betanin 6′-*O*-sulfate), neobetanin (5-*O*-*β*-D-glucopyranosylneobetanidin), amaranthin, lampranthin I (4-coumaroylbetanin), and lampranthin II (feruloylbetanin) (Alard et al., 1985; Bokern et al., 1991; Jackman and Smith, 1996). Feruloylamaranthins have earlier been reported in cell suspension cultures of *Chenopodium rubrum* (Strack et al., 1988).

In our earlier study on red beetroot peel constituents (Kujala et al., unpublished results), ferulic acid was found but its conjugates were not. This, however, may be the result of the degradation of conjugates during the separation used.

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Received for review April 26, 2000. Revised manuscript received August 25, 2000. Accepted August 25, 2000. This work was supported by the Finnish Ministry of Agriculture and Forestry and by Tekes Technology Development Center.

JF000523Q